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RESEARCH ARTICLE

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Reduction of four bis-azo dyes by pig liver microsomal fraction in anaerobic conditions

Paola Faraoni[1,2] | Alessio Gnerucci[1,2,3] | Serena Laschi[2] | Francesco Ranaldi[1,2]

[1]Department of Experimental and Clinical Biomedical Sciences "Mario Serio," University of Florence, Florence, Italy

[2]"Nanobiosens" Joint Lab, University of Florence, Florence, Italy

[3]Department of Physics and Astronomy, University of Florence, Florence, Italy

Correspondence
Paola Faraoni, Department of Experimental and Clinical Biomedical Sciences "Mario Serio," University of Florence, Viale G. Pieraccini, 6, 50139 Florence, Italy.
Email: paola.faraoni@unifi.it

Abstract

The study of azo dyes degradation products and their harmfulness is an important topic because their diffusion in the environment is still a serious problem. We investigated the reduction by pig liver microsomal fraction of four bis-azo dyes, Acid Red 73, Acid Red 150, Direct Red 24, and Direct Red 28, in anaerobic condition. These complex molecules, different for position/nature of substituent groups and for the group interposed between the two azo bonds, allowed to investigate the correlation between substrate molecular structure and action of a subcellular fraction representative of the xenobiotic degradation by a living organism and the consequent possible appearance of harmful reaction products. Dyes were first carefully purified to eliminate interferences in the enzymatic assays. The reduction products were identified by gas chromatography/mass spectrometry. Acid Red 73 and Acid Red 150 were markedly reduced with production of harmful aromatic amines, while Direct Red 24 and Direct Red 28 were neither reduced to their synthesis amines nor to other amines. These results suggest that azo dyes with a benzidine derivative group are not degraded by microsomal fraction and therefore molecules with a similar structure could be classified with a lower risk index not producing any harmful catabolic product.

KEYWORDS
anaerobiosis, aromatic amines, bis-azo dyes, pig liver microsomes, xenobiotic degradation

1 | INTRODUCTION

From the twentieth century, with the increase of the knowledge of chemistry and the development of organic compounds synthesis methods, a wide diffusion of artificial colorants occurred, and they supplanted natural pigments.

Among the dyes, the more diffused are the azo dyes, so-called for the presence of at least one azo bond (-N = N-). These dyes have been and still are widely used for their high brightness (high molar extinction value) and for their ease in binding to the substrate in the textile, food, cosmetic, pharmaceutical industries, by tattooists and also for cytological and histological staining.

More than 10 000 azo dyes are known, which have one or more azo bonds linked to different chemical groups and which cover the entire range of colors of the visible spectrum. At least 2000 of these dyes are actually commonly used.[1]

The widespread use of these substances determines in the first instance a problem in terms of environmental pollution. Their discharges in rivers and lakes cause both a loss of water transparency with a decrease in light penetration, and a consequent reduction in phytoplankton photosynthesis. An additional problem is the diffusion of the polluting substances from the waters to the neighboring soils.[2]

These dyes are highly resistant to the action of detergents, sun exposure and temperature changes. The degradation of these pigments involves the breakdown of the azo bond, which is not present

Paola Faraoni, Alessio Gnerucci, and Serena Laschi contributed equally to this work.

in nature, through a reduction process catalyzed by an azoreductase complex. This complex is found in mammalians, especially in liver, bowel (with an important role of bacterial flora) and in the skin microflora.[3–8]

The toxicity of these molecules is not related to their intrinsic nature itself, but to the possible degradation products that originate from the breakdown of the azo bonds operated by azoreductase (aromatic amines, benzidine, aniline, toluidine whose mutagenicity and carcinogenicity are well documented).[9–14] Tests on cell lines and laboratory animals showed chromosomal alterations (clastogenic effect)[15–17] and development of neoplasms (for example in bladder and liver)[1] after administration of these substances. Moreover, their ability to interfere with melanin production and to determine hypopigmentation as they alter the functionality of tyrosinase has also been highlighted.[18]

As a result of this evidence, the legislation of many countries has banned and/or limited their use. At first their use was regulated in Germany with the 1995 announcement[19] and then in the entire European community,[20] with even recent updates, such as the new instructions and classifications of the European Chemicals Agency (ECHA),[21] as well as non-European countries such as Australia.[22]

However, the use and spread of azo dyes remain very high. Even if many of them are banned in many countries, they continue to be produced and used in other ones. Globalized commerce and inadequate controls still allow their easy availability. Furthermore, these dyes are released in the environment through the massive fabric recycling effort. Considering the consequences in the environment and for human health, high attention is paid to the possible methods to decrease the environmental impact of their diffusion.[23–25]

The possibility of biodegradation of these dyes by specific microorganisms has been considered because various bacteria can metabolize the azo bond.[26–29] Other methods to reduce environmental harmfulness include the use of physical and chemical techniques which, however, have a high impact due to the considerable quantities of chemicals used and produced by these processes.[30,31] Furthermore, these techniques are also very expensive.

Our work is aimed at the study of the metabolism of some azo dyes extensively used in the textile dyeing industries in experimental conditions similar to enzymatic degradation in an in vivo situation.

The enzymatic reduction of the azo bond by pig liver microsomes in anaerobic condition was investigated in order to evaluate the possible formation of products dangerous to living organisms and to correlate it with the substrate molecular structure.

The study of the enzymatic reduction reaction mechanism and its kinetics properties, despite providing fundamental information, is beyond the aims of this work that focuses, instead, on the investigation of the action of a subcellular fraction, representative of the xenobiotic degradation, occurring in a living organism and consequently on the assessment of the possible appearance of harmful reaction products.

We identified the catabolic products of the degradation of four dyes all characterized by the presence of two azo bonds (bis-azo compounds). Acid Red 73 and Direct Red 24 were used to dye wool and cotton and are now banned, Direct Red 28 is used for coloring wool fibers and also as pH indicator, Acid Red 150 is a dye used in histology. These dyes differ in presence or absence and in the position of different chemical groups and in the chemical structure of the group interposed between the two azo bonds (Figure 2). These molecules were chosen to be representative of chemical structures characteristic of many bis-azo dyes and can therefore give important information about their possible degradation and products as well as become a starting point for deeper investigation about the kinetics and action mechanisms of the enzymatic complex.

2 | MATERIALS AND METHODS

2.1 | Chemicals

NAD^+ and $NADP^+$ coenzymes, glucose dehydrogenase, mutarotase, glucose-6-phosphate dehydrogenase, glucose oxidase, *p*-dimethylaminoazobenzene (DAB), β cyclodestrin, TRIS-maleate buffer, Hepes, sucrose, glucose, glucose-6-phosphate, and analytical standards (hexachlorobenzene and Fenchlorphos) were purchased from Merck. Acid Red 73 (C.I. 27290, Brilliant Crocein, 3-hydroxy-4-[[*p*-(phenylazo)-phenyl]azo]-5,7-naphthalene disulfonate disodium salt) and Direct Red 24 (C. I. 29185, Direct Fast Scarlet 4BA, 3-[(*o*-metoxyphenyl)azo]-3'-[(4-sulfo-*o*-tolil)azo]-7,7'-ureilenebis[4-hydroxy-2-naphthalensulfonate] trisodium salt) were obtained from ACNA (Aziende Colori Nazionali Affini, Italy). Direct Red 28 (C. I. 22120, Congo Red, 3,3'-[4,4'-biphenylylenebis-(azo)]bis[4-amino-1-naphthalene sulfonate] disodium salt) and Acid Red 150 (C. I. 27190, Ponceau SS, 3-hydroxy-4-[[*p*-(phenylazo)-phenyl]azo]-2,7-naphthalene disulfonate disodium salt) were provided by Merck.

2.2 | Purification and analysis of the dyes

All the azo dyes tested (except DAB) needed to be purified due to the presence of unknown impurities. Only for Direct Red 28 a purification procedure was found in the literature.[32] This method was used as a reference for the purification of the other dyes applying with some modifications.

To carry on the purification, 2 g of each dye were mashed with few drops of water (about 1 mL). Then, 199 mL of water were added (1% w/v) and the mixture was left under stirring overnight. This step allowed the solubilization of the contaminant salts.

The suspension was then filtered, and the dye recovered on the filter was vacuum-dried. The obtained powder was weighed, mashed with about 1 mL of water, solubilized in water (1% w/v), boiled for 20 min in a water bath equipped with a cooling system to avoid sample drying, and then filtered. The dye present in the filtrate was crystallized adding an equal volume of ethanol and keeping the solution at 4°C overnight. Then, after filtration on a sintering glass filter (G3), the crystals were rinsed with cold water or absolute ethanol. The dye was then vacuum-dried and crushed in a mortar.

This procedure was repeated two times to obtain a good degree of dyes purity, evaluated by thin layer chromatography (TLC, silica gel plate, elution by a mix of butanol saturated with a solution of 2 N NH_3 and ethanol, 3:1) and centesimal analysis. For TLC analysis the dyes were used in 1 mM water solution.

Stock solutions of each purified dye (0.1 mM in water) were prepared. The 0.1 mM stock solution of DAB, insoluble in water, was prepared in dymethylsulfoxide (DMSO).

2.3 | Preparation of pig liver microsomal fraction

After removing the connective tissue, liver from pig, not treated with phenobarbital,[33] was washed with physiological solution and then homogenized in a glass/Teflon potter in a solution containing 0.025 mM Tris-maleate, pH 7.2, 1 mM $MgCl_2$, 0.25 M sucrose (1:10 w/v) at 4°C. The homogenate was then filtered through filter paper and then centrifuged at 600 × *g* for 10 min. After filtration by Miracloth membrane (Millipore), the supernatant was centrifuged at 12 000 × *g* for 20 min. Then, the internal membranes pool (microsomal fraction) was precipitated by centrifugation at 105 000 × *g* for 35 min and, after supernatant removal, washed twice with physiological solution and resuspended in physiological solution if immediately used. In case of storage, aliquots at a protein concentration of 10 mg mL^{-1} in 0.25 M sucrose solution were prepared and then frozen at −80°C. As marker of microsomes activity, glucose-6-phosphatase (E.C. 3.1.3.9) was assayed[34] by monitoring in continuum the NAD^+ reduction by spectrophotometry at 340 nm.[35]

The assay mixture (final volume of 1 mL) included 50 mM Hepes, 8-unit glucose dehydrogenase (E.C. 1.1.1.47), 0.3-unit mutarotase (E.C. 5.1.3.3), and 2 mM NAD^+. The reaction was started by adding the substrate (20 mM glucose-6-phosphate).

2.4 | Dye enzymatic degradation

The enzymatic reduction of the dyes was carried out by incubating pig liver microsomal fraction and the dye in anaerobic condition (to eliminate the possibility that oxygen inhibition could represent a further reaction parameter)[36–38] and assayed spectrophotometrically, following the decrease of the absorbance at the wavelength corresponding to maximum absorbance of dye continuously, according to Zbaida et al.[39] with slight modifications.

In fact, the biological functionality of microsomal fraction is strictly correlated to the presence of benzene azoreductase (E.C. 1.6.6.7).

The reaction mixture (final volume of 10 mL) is reported in Table 1.

Glucose-6P-dehydrogenase (E.C. 1.1.1.4.9) and glucose oxidase (E.C. 1.1.3.4) were suspended in 100 mM Hepes, pH 7.4.

In order to remove oxygen, 10 mL of the reaction mixture were placed in a vial with a two-way valve, in which nitrogen was bubbled for 10 min at 4°C.

The reaction mixture without $NADP^+$ was used as reference sample for the evaluation of no enzymatic reduction (i.e., nonspecific reduction).

TABLE 1 Reaction mixture for the enzymatic assay.

	Assay concentration
100 mM Hepes, pH 7.4	50 mM
300 mM $MgCl_2$	10 mM
40 U mL^{-1} Glucose-6P-dehydrogenase	2 U mL^{-1}
360 U mL^{-1} Glucose oxidase	20 U mL^{-1}
300 mM Glucose	60 mM
400 mM Glucose-6P	5 mM
1.5% β-Cyclodestrine	0.03%
10 mg prot. tot. mL^{-1} Microsomes	0.5 mg mL^{-1}
0.1 mM Dye	10 μM
25 mM $NADP^+$	0.5 mM
H_2O (to a final volume of 10 mL)	

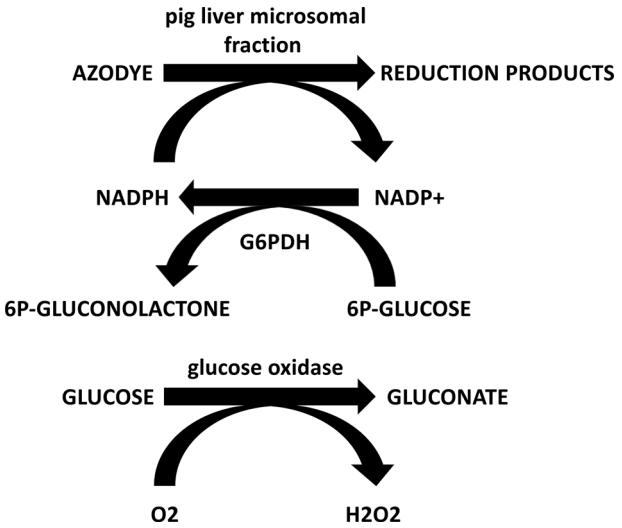

FIGURE 1 Scheme of the dye reduction reaction by pig liver microsomal fraction. The reaction starts by adding $NADP^+$, the anaerobic condition is guaranteed by glucose oxidase; the reducing power (NADPH) was generated by glucose-6P-dehydrogenase.

For each dye, six vials were prepared, with three representing the reference (mixture assay without $NADP^+$) and the other three the samples (with $NADP^+$). The samples and related references were incubated at 37°C for 6 h. The reaction was stopped by fast cooling at 0°C. Samples were subsequently stored at −20°C and thawed at the time of analysis in order to determine aromatic amines content.

The reaction started by adding $NADP^+$ in anaerobic condition and proceeded according to the scheme shown in Figure 1: the glucose oxidase/glucose system guaranteed anaerobic condition and the glucose-6P-dehydrogenase/glucose-6-phosphate/NADP+ system supplied reducing power (NADPH) for breaking azo bonds. β-Cyclodextrin was used in the assay to better solubilize the dyes.

It is important to remark that the reaction was performed until completion (the absorbance of reaction mixture no longer changes).

2.5 | Determination of protein concentration

Protein concentration was determined by the Bradford method.[40] All the spectrophotometric measurements were performed by a double-beam spectrophotometry (Shimadzu UV-VIS-2100).

2.6 | Aromatic amines analysis

Ten milliliters of of samples and corresponding references were placed on a 25 × 80 mm column packed with diatomaceous earth together with 1 mL of an internal standard (hexachlorobenzene). These were left to adsorb for 20 min and then eluted by 80 mL of methyl-*t*-butyl-ether. After the addition of another internal standard (Fenchlorphos), the eluate was concentrated in a rotary evaporator (Buchi 461) to a volume of about 0.5 mL, and then diluted up to 2 mL using the eluent.

The qualitative and quantitative analysis of aromatic amines was obtained by gas chromatography-mass spectrometry (GS-MS, Varian 3400 CX gas chromatograph coupled with a mass spectrometer Varian Saturn 2 4D, equipped with Varian 8800 CX autosampler). A DB-35MS fused silica capillary column (length: 30 m, internal diameter: 0.25 mm, film thickness: 0.25 μm, Agilent) was used and He was the carrier gas used with a flow rate of 1.0 mL min^{-1}. The sample was injected in splitless mode and the injected volume was 1 μL. The GC oven temperature was maintained at 55°C for 5 min; it was increased to 300°C at a rate of 10 °C min^{-1} and kept at this value for 6 min. The temperatures of the injector, transfer line, and ion source were 260°C, 240°C, and 240°C, respectively. The MS was operated in the electron ionization mode at 70 eV.

3 | RESULTS AND DISCUSSION

In Figure 2, the chemical structure of the investigated azo dyes is shown. All of them are characterized by a molecular structure more complex compared to that of many molecules already studied in the literature, such as DAB, with the presence of two azo bonds. They, however, differ for the presence/absence/position of chemical groups with electron attractor and/or electron donor proprieties and with different steric hindrance. The group interposed between the two azo bonds has a different chemical structure. Acid Red 73 and Acid Red 150 present a single benzene ring, whereas Direct Red 28 presents a diphenyl group and Direct Red 24 presents a particularly complex structure: the two azo groups are spaced by urea substituted with two naphthalene rings on both nitrogen atoms.

The nature of the observed reaction products can give therefore indication on which of the two azo bonds (marked with A and B in Figure 2) was broken.

The first fundamental step of our study was the purification of azo compounds in order to carry out the enzymatic reduction without foreign molecules, which can alter the enzymatic complex activity of liver microsomal fraction: the compounds available on the market are highly impure, mainly those used in the textile fiber dyeing sector. Impurities are mainly inorganic compounds (i.e., Na_2SO_4) used for cutting the dyes, the synthesis intermediates and also other coloring substances useful to give the dye the required shade. It is very difficult to obtain information from literature on coloring matter used for dyeing chemistry because of old industrial patents. Only for Direct Red 28 we had information about its purification,[32] even if this compound is sold by the manufacturer at a declared purity of 90%.

The absence of any other coloring substances in the purified dyes was ascertained by thin layer chromatography. The centesimal analyses of purified compounds gave remarkable results (Table 2).

From the absorption spectra, we deduced that the maximum absorbance of these dyes is in a restricted range of around 500 nm. At these wavelengths, the corresponding molar extinction values were then determined (Table 3).

Through the absorbance decrease at the maximum absorbance wavelength, evaluated spectrophotometrically, we have investigated the possibility that the four purified dyes (and DAB, as positive control) could be degraded from pig liver microsomal fraction. Acid Red 73 and Acid Red 150 showed this decrease in the absorbance peak.

It is important to report that this was not observed for nonpurified Acid Red 150. This result underlines the importance of the dyes purification protocol.

Direct Red 28 and Direct Red 24 also did not show this absorbance reduction.

We want to remark that this spectrophotometrical measurement could not indicate if the azo bond was broken, but a generic chemical modification of the dyes in the reaction mixture. The qualitative and quantitative GC-MS analyses of the amines extracted from reaction mixture (Figures 3–6) allowed the identification of the reaction products and therefore, confirmed whether the azo bonds were broken or not.

Figure 3 shows the gas chromatogram of the reference solution containing the aromatic amines that were sought as reaction products in the samples (aniline, *p*-phenylendiamine, *p*-aminoazobenzene, benzidine, and *o*-toluidine).

The analysis of the DAB's reaction mixture showed in the mass spectrum of sample solution (Figure 4A) a peak corresponding to aniline, with respect to reference solution (Figure 4B); this result was confirmed by the molecular ion present in the mass spectrum. In our study DAB was used as control and reference for the comparison with the widely available literature.[41–45]

Acid Red 73 (Figure 5) and Acid Red 150 (Figure 6) showed a similar behavior and presented peaks with retention times consistent to those of aniline, *p*-phenylendiamine, and *p*-aminoazobenzene.

The chromatograms related to reference and reduced samples of Direct Red 28 and Direct Red 24 (data not shown) are very similar and do not present peaks with retention times consistent to those of standard attempted aromatic amines thus confirming the results of the spectrophotometrical evaluation that indicate that these two dyes were not reduced.

Acid Red 73 and Acid Red 150 are both reduced as both the spectrophotometric assays and the GS/MS analysis indicate. In Figure 7, the chemical structure of these two dyes as well as of the aromatic

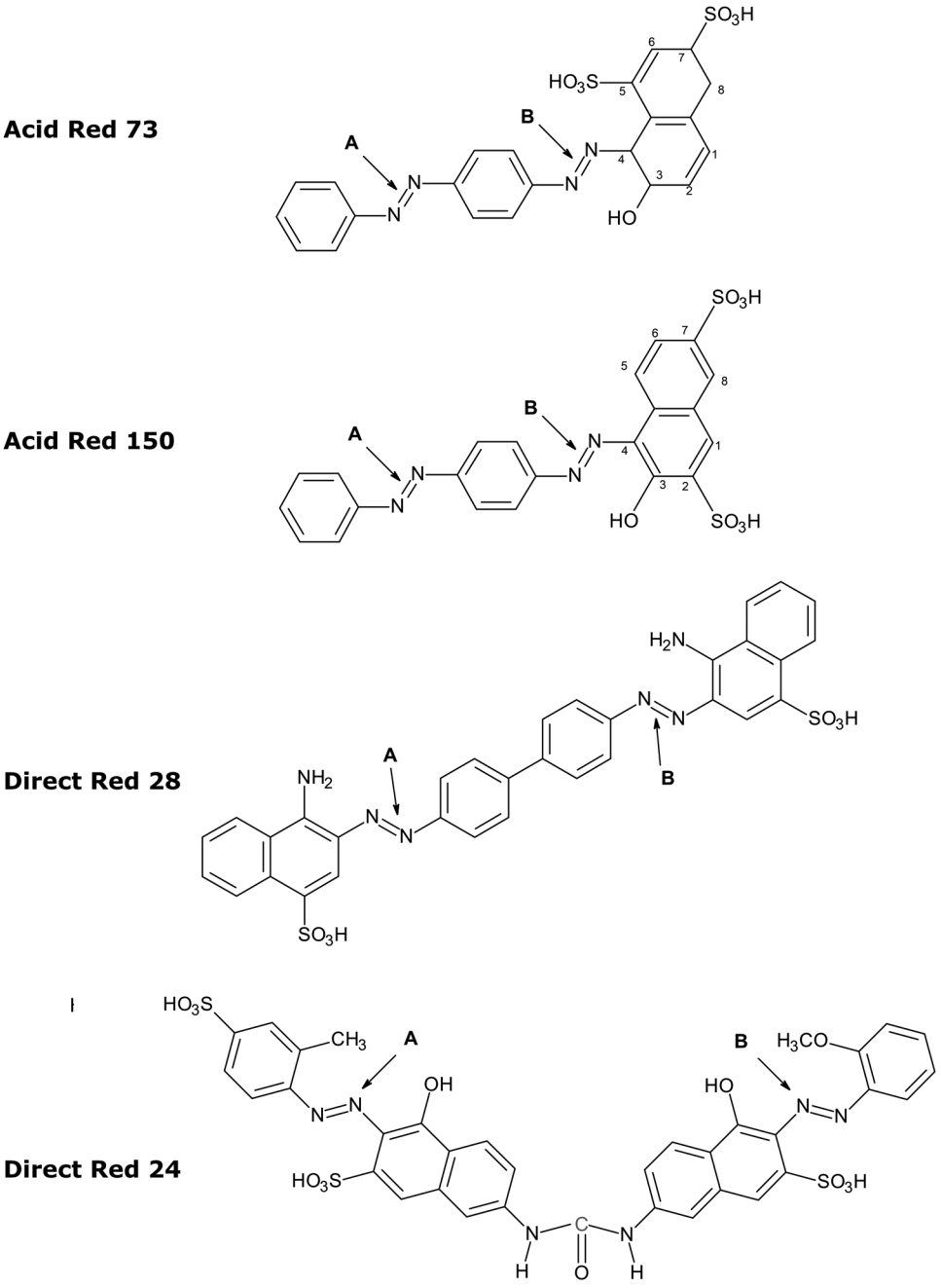

FIGURE 2 Chemical structures of the four investigated dyes: the two azo bonds in each molecule are marked with the letters A and B.

TABLE 2 Centesimal analysis of azo dyes.

	Composition (%)	
Azo dye	Theoretical[a]	Measured
Acid Red 73	C 47.49, N 10.07, H 2.54 ($C_{22}H_{14}N_4O_7S_2Na_2$)	C 47.48, N 10.07, H 2.54
Acid Red 150	C 47.49, N 10.07, H 2.54 ($C_{22}H_{14}N_4O_7S_2Na_2$)	C 47.48, N 10.07, H 2.54
Direct Red 24	C 46.57, N 9.31, H 2.79 ($C_{35}H_{25}N_6O_{13}S_3Na_3$)	C 46.56, N 9.31, H 2.79
Direct Red 28	C 55.17, N 12.06, H 3.18 ($C_{32}H_{22}N_6O_6S_2Na_2$)	C 55.17, N 12.07, H 3.19

[a]The theoretical percentages are calculated from the dye molecular formula.

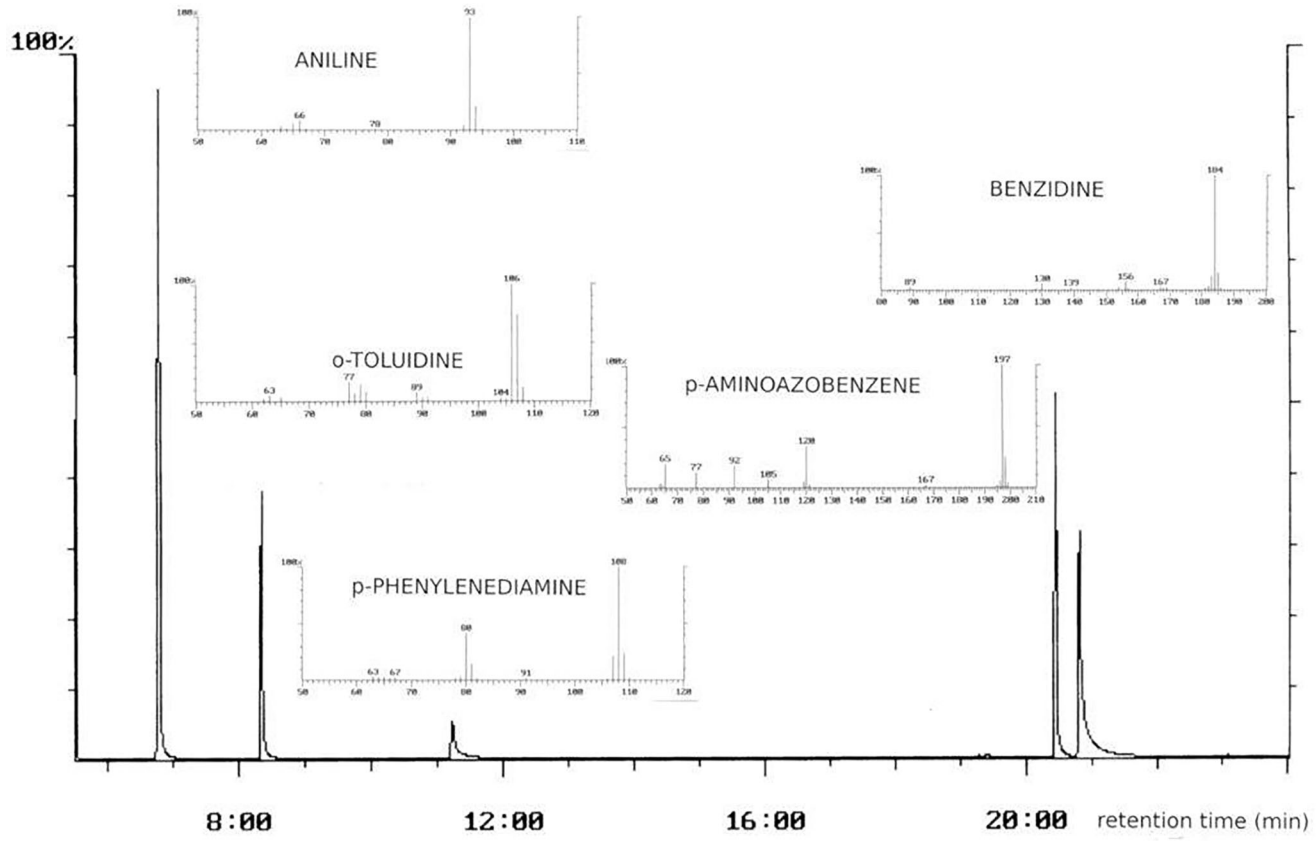

FIGURE 3 Gas chromatogram of the standard aromatic amines sought in the reaction mixture as putative products of enzymatic reduction of tested dyes by pig liver microsomal fraction. The chromatogram peaks of each aromatic amine are matched with the corresponding mass spectrum (aniline, *p*-phenylendiamine, *p*-aminoazobenzene, benzidine, and *o*-toluidine).

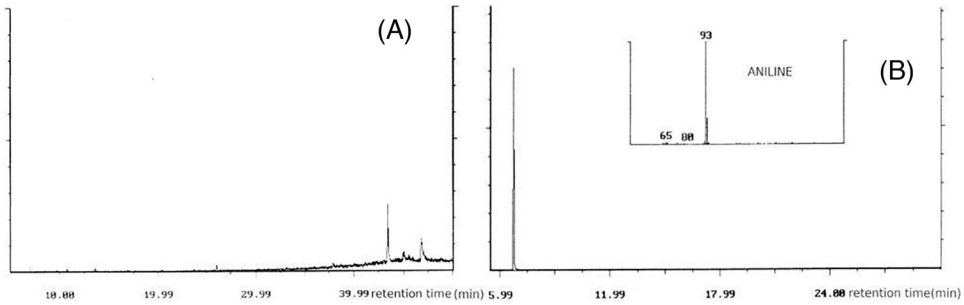

FIGURE 4 Gas chromatogram of aromatic amines as products of DAB enzymatic reduction. The chromatogram of DAB sample solution is matched with the mass spectrum of peaks corresponding to produced amines (aniline). A) reference solution, B) sample solution.

TABLE 3 Wavelengths corresponding to the maximum absorbance and relative molar extinctions for the studied dyes.

Azo dye	$\lambda_{max\ abs}$ (nm)	Molar extinction value $\varepsilon\lambda_{max\ abs}$ ($mM^{-1}\ cm^{-1}$)
Acid Red 73	507	42.9
Acid Red 150	514	46.9
Direct Red 24	495	52
Direct Red 28	494	28

amines detected are shown. The detection of *p*-aminoazobenzene, *p*-phenylendiamine, and aniline with a ratio of 10:1:1 can give the indication that preferentially the first reduced azo bond is the one linked to the substituted naphthalene ring (marked with B in Figure 7).

It should be noted that often the breaking of the two different azo bonds present in the same molecule does not occur simultaneously, but can happen in more progressive steps, as it is reported in the literature, for example, in case of the bis-azo dye Brilliant Black BN.[46] For this dye, the azo bond linked to the benzene ring is reduced first with the

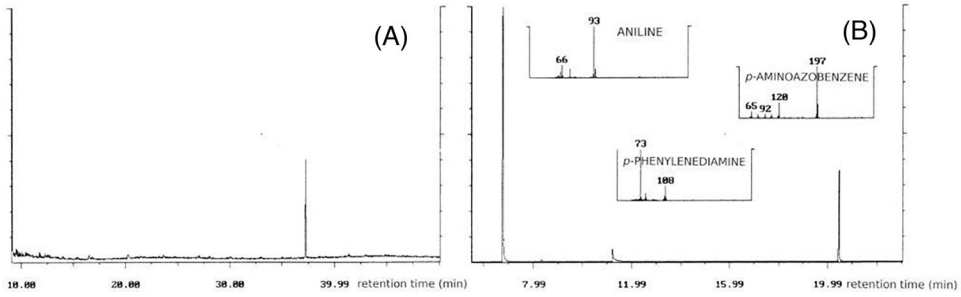

FIGURE 5 Gas chromatogram of aromatic amines as products of Acid Red 73 enzymatic reduction. The chromatogram of Acid Red 73 sample solution is matched with the mass spectrum of peaks corresponding to produced amines (aniline, *p*-phenylendiamine, and *p*-aminoazobenzene). A) reference solution, B) sample solution.

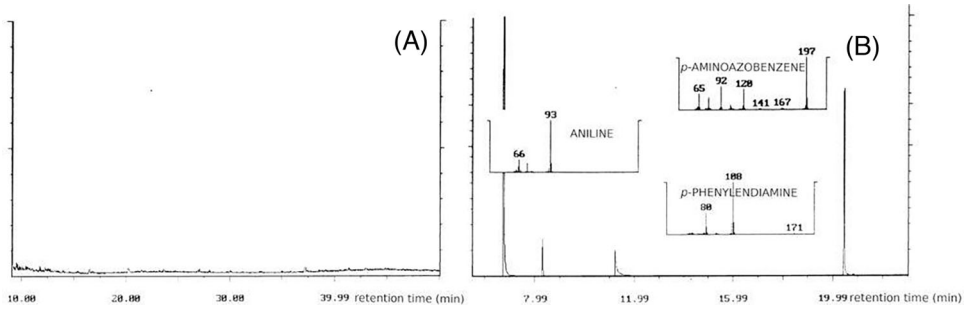

FIGURE 6 Gas chromatogram of aromatic amines as products of Acid Red 150 enzymatic reduction. The chromatogram of Acid Red 150 sample solution is matched with the mass spectrum of peaks corresponding to produced amines (aniline, *p*-phenylendiamine, and *p*-aminoazobenzene). A) reference solution, B) sample solution.

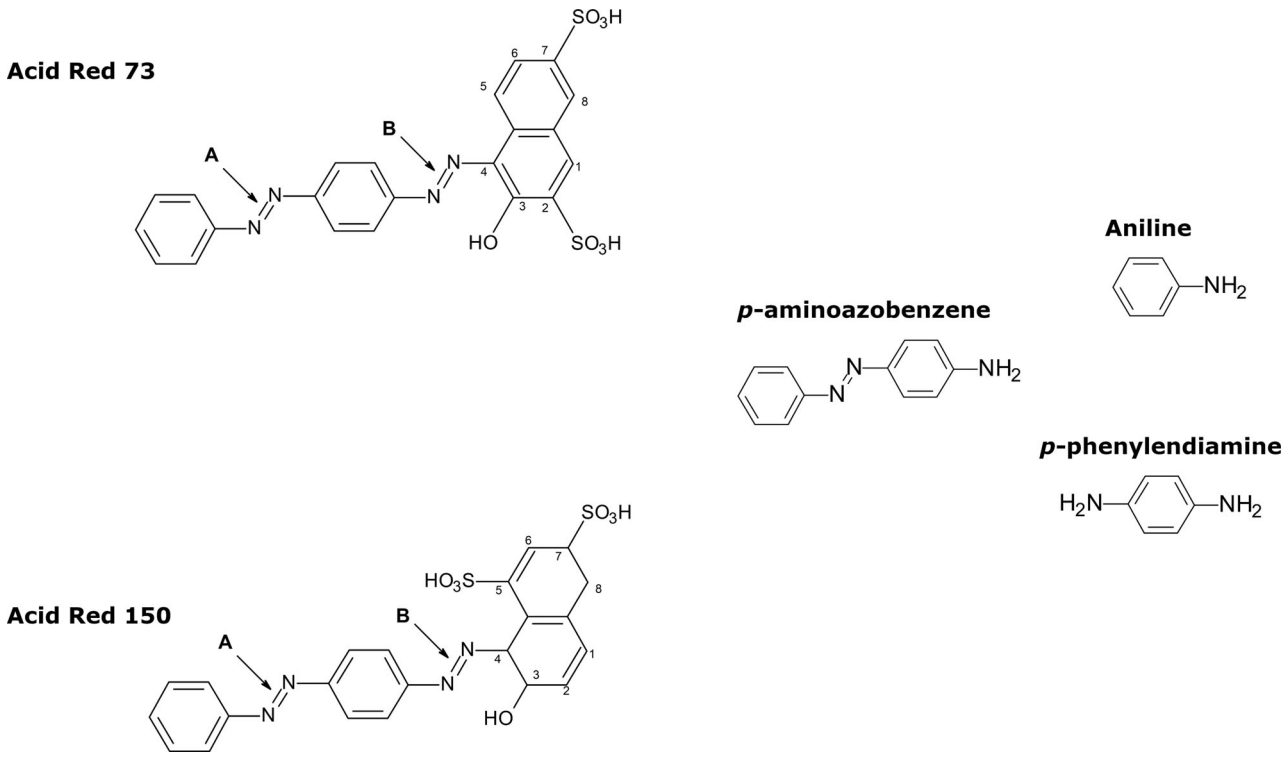

FIGURE 7 Chemical structure of Acid Red 73, Acid Red 150 (on the left, the two azo bonds in each molecule are marked with the letters A and B) and of the aromatic amines detected (on the right, aniline, *p*-phenylendiamine, and *p*-aminoazobenzene) after the enzymatic reduction by pig liver microsomes.

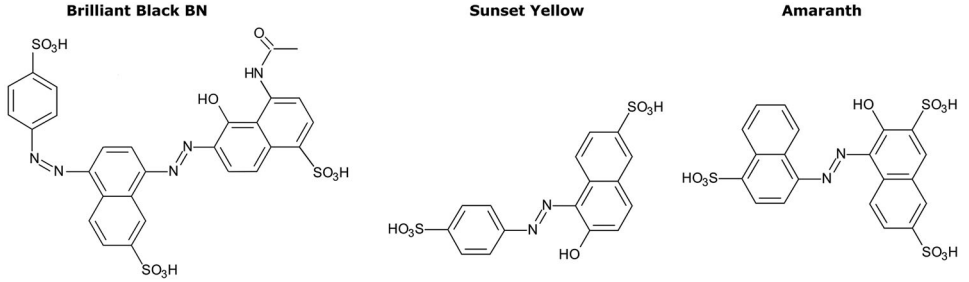

FIGURE 8 Structure formula of Brilliant Black BN (C. I. 28440), Sunset Yellow (C. I. 15985), and Amaranth (C. I. 16185).

formation of an orange intermediate containing the other azo bond, which is subsequently reduced (production of two additional aromatic amines).

The breaking of azo bond A in Acid Red 73 and Acid Red 150 could result in aniline and then *p*-phenylendiamine by subsequent reduction of the azo bond in 3-hydroxy (4-(4-amino-1-phenyl)azo)-5,7-naphthalendisulfonate sodium (for Acid Red 73), or the analogous 2,7-disulfonated (from Acid Red 150), similarly to what happens in the reduction of other azo dyes such as Sunset Yellow (Figure 8).[47,48] This reduction process does not explain the presence of *p*-aminoazobenzene in the products.

The simultaneous breaking of azo bonds A and B also could not explain the detection of *p*-aminoazobenzene in such an amount.

As a consequence of our observations, it can be thought that these two bis-azo dyes are reduced in a step-by-step process that first involves the breaking of bond B with the production of 3-hydroxy 4-amino-5,7-naphtalendisulfonate (for Acid Red 73), or 3-hydroxy 4-amino-2,7-naphtalendisulfonate (for Acid Red 150) and *p*-aminoazobenzene, whose azo bond is subsequently reduced with the production of aniline and *p*-phenylendiamine as mentioned by Zissi et al.[49]

This preferential reduction of the azo bond connected on one side to a benzene ring and on the other to naphthalene is in accordance with what happens in Black Brilliant Black.[46] Moreover, the result of the reduction of Acid Red 73 is in line with the reduction of Amaranth that has a very similar structure (Figure 8).[50]

From the studies of Zbaida and Levine,[45] we would expect *p*-aminoazobenzene with an electron donor group in the para position to the azo bond to be completely broken into its parent amines. On the contrary, according to our results, the equilibrium of the reduction reaction of *p*-aminoazobenzene is much more shifted toward this reagent rather than to the products (aniline and *p*-phenylenediamine). We believe that kinetic studies are needed to clarify this point. In any case, the presence of *p*-aminoazobenzene after reduction confirms the harmfulness of these two dyes.

It is worth noting that the breaking of the azo bonds can produce the synthesis reagent amines, but also to different amines. In Acid Red 114, for example, chemical reduction of azo bonds generates synthesis reagent amines, while in Acid Orange 7, 1-amino-naphthol, which is not a synthesis reagent, is obtained.[51] The case of *p*-aminoazobenzene discussed above in our results confirms this possibility. Therefore, a dye synthesized from not harmful amines, if reduced, can give rise to mutagenic and carcinogenic aromatic amines.

The experimental results indicate that Direct Red 28 is not degraded by pig liver microsomes. This observation seems to confirm a previous study about this dye by Martin and Kennelly.[50] Their results indicated that the presence of a diphenyl group interposed between two azo bonds is not suited to the action of the enzymatic complex.

Our work differs from that of Martin and Kennelly[50] since the microsomal fraction that we used was obtained from liver of pigs not treated with phenobarbital (this treatment was common in this kind of studies, because it induces an overexpression of cytochrome P-450).[33] Nevertheless, our results, indicating that this dye is not reduced, seem to confirm their hypothesis.

Direct Red 24 too seems insensitive to the action of pig liver microsome, nevertheless its molecule has a moiety (azo bond connected on one side to a benzene ring and on the other to a naphthalene ring) with structure similar to Sunset Yellow,[50] Acid Red 73, and Acid Red 150. Most likely the presence of the substituted ureidic group is a geometric factor that prevents the action of degradation.

In our opinion, the meaning of this work is to verify in a pragmatic way if such bis-azo dyes are reduced by a microsomal fraction representative of the in vivo situation. As considered by Martin and Kennelly,[50] a much larger sample of dyes needs to be tested to get a more extensive knowledge of the correlation between chemical structure of azo dyes and their possible enzymatic reduction. This work contributes to increase the number of tested dyes.

Moreover, we want to remark that our methodological approach is in agreement with other works present in the literature for example as given by Franco et al.,[52] although they have studied the reduction of different azo dyes by rat liver microsomes instead of pig liver microsomes and in aerobic condition.

4 | CONCLUDING REMARKS

In this work, we investigated the reduction of four bis-azo dyes by pig liver microsomal fraction obtained from animals not treated with phenobarbital. The chosen dyes (Acid Red 73, Acid Red 150, Direct Red 24, and Direct Red 28) are characterized by a complex structure with two azo bonds and differ in position of the azo bond and in the chemical groups present and therefore represent an important benchmark

for the study of the enzymatic complex action on different molecular structures.

We observed that Acid Red 73 and Acid Red 150 were markedly reduced. The detection in the reaction products of aniline, *p*-phenylendiamine, and *p*-aminoazobenzene gives an indication, supported by other results in the literature, that the breaking of azo bonds occurs stepwise with the formation of *p*-aminoazobenzene subsequently reduced to aniline and *p*-phenylendiamine.

On the other hand, Direct Red 24 and Direct Red 28 are neither reduced to their synthesis amines nor to other amines, and this suggests that probably other dyes with similar structure (i.e., the dyes with a group derived from benzidine interposed between the two azo bonds) are not reduced with the formation of harmful aromatic amines and could be therefore classified with a lower hazard index.

We also underline the importance of a purification process of azo dyes subjected to enzymatic reduction to avoid unwanted biases of the studied reactions due to unknown impurities. The fact that not purified Acid Red 150 was not reduced by the microsomal fraction while the purified was confirms this observation.

Despite the study of the enzymatic reduction reaction mechanisms and kinetics is beyond the aims of this work, our results can surely provide more information about the correlation between the chemical structure of azo dyes and their possible enzymatic reduction, also in the context of molecular dynamic simulations studies.

Finally, the degradation action occurring in bowel and skin with their microflora and the role of enterohepatic circulation should not be neglected for a more complete understanding of the catabolism of these dyes.

AUTHOR CONTRIBUTIONS

Paola Faraoni: methodology; validation; writing—original draft; writing—review & editing. Alessio Gnerucci: methodology; validation; writing—original draft; writing—review & editing. Serena Laschi: methodology; validation; writing—original draft; writing—review & editing. Francesco Ranaldi: conceptualization; investigation; methodology; writing—original draft; writing—review & editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interest.

DATA AVAILABILITY STATEMENT

Research data are not shared.

ORCID

Paola Faraoni https://orcid.org/0000-0003-3967-2886

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